Generation of Gelatin-Based Antibiotic-Eluting Conjugates for the Prevention of Surgical Site Infections

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Abstract

Surgical site infections (SSIs) following major surgeries are a growing concern in the healthcare industry. These infections lead to lengthened hospital stays, additional surgical procedures, prolonged antibiotic use, and increased patient morbidity. Current prevention methods include oral administration of antibiotics before surgery, coating of antiseptic agents on incision sites and operating utensils, and systemic administration of antibiotics during surgery. With widespread delivery of antibiotics, there is an increased likelihood of further expansion of antimicrobial resistance. The development of a localized antibiotic-eluting hemostatic agent as a preventive measure will likely improve the efficiency and efficacy of antimicrobial prophylaxis and subsequently reduce the occurrence of SSIs. Therefore, the current investigation identified a methodology to produce a vancomycin-linked hemostatic agent through liquid-phase peptide bond formation. Type B gelatin was incubated with vancomycin and the cross-linking agent 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide (EDC) to allow for internal cross-linking of gelatin and conjugation of gelatin with vancomycin. Infrared (IR) spectroscopy, high pressure liquid chromatography (HPLC), and UV-Vis spectrometry confirmed that vancomycin released from the loaded hemostatic agent maintained its structural integrity following the cross-linking procedure. Additionally, the activity of eluted vancomycin was analyzed through Kirby-Bauer and microdilution assays, and MTT assays assessed toxicity of the released antibiotic. The method described here allows for the production of a biocompatible and active vancomycin-eluting hemostatic agent for the prevention of SSIs. Beyond vancomycin, this methodology was expanded to produce hemostatic agents that elute daptomycin, ceftazidime, and ceftibuten. Incorporation of this technology into post-operative procedures may expedite surgical protocols, limit cost burdens, and decrease mortality rates associated with SSIs.

Introduction

Surgical Site Infections

Surgical site infections (SSIs) are a major concern in the healthcare industry that lead to lengthened hospital stays, additional surgical procedures, prolonged antibiotic use, and increased patient morbidity (*1-2*). Although infections are often remitted, they increase medical costs and reduce functional prognosis of patients after surgery (*3-5*). It is estimated that SSIs occur during 2% to 13% of spinal surgeries and periprosthetic joint infections will occur in up to 80,000 patients per year in the United States by 2030 creating a cost burden up to $4 billion annually (*6-8*). Through epidemiological studies, several risk factors for SSIs have been identified. Systemic patient-related factors including diabetes, malnutrition, cigarette use, and steroid use increase the risk of infection (*9*). Several preventive methods have been considered effective in limit the occurrence of SSIs including surgical hand preparations, post-discharge surveillance, postponing elective surgeries in the case of an existing infection, and antimicrobial prophylaxis (*7, 9*).

In the majority of SSI cases, the responsible pathogens originate from the patient's endogenous flora (*9*). The pathogens most commonly found responsible are *Escherichia coli, S*taphylococcus *aureus*, coagulase-negative staphylococci, and *Enterococcus.* Beyond endogenous sources, pathogens may originate from the operating room environment, members of the surgical team, and instrumentation (*9*). These are predominately gram-positive aerobes such as staphylococci and streptococci. Likely due to the widespread use of antibiotics and increased number of immunocompromised patients, an increasingly high number of these SSIs originate from Methicillin-Resistant *Staphylococcus Aureus* (MRSA) (*9*). From 2012 to 2017, the mortality rate of patients infected by MRSA averaged 29% (*10*) but may be as high as 74% for vulnerable populations (*11*).

Antibiotics in the Prevention of Surgical Site Infections

Antimicrobial prophylaxis, the use of antibiotics to target pathogens and prevent infection, has become standard practice after surgery (*2, 8, 12*). Cefazolin and other cephalosporins are considered sufficient to be used in antimicrobial prophylaxis to target Staphylococcus aureus (*13-14*). However, due to increased rates of MRSA induced SSI, vancomycin and other glycopeptides have been more widely used (*14-15*). Vancomycin's minimal inhibitory concentration (MIC) varies somewhat based on the strain but reaches a maximum at 2 µg/mL and a minimum at 0.125 µg/mL (*16-19*).

Although vancomycin selects exclusively for gram-positive bacteria, gram-negative infections rarely occur after clean surgery and typically appear to be derived from beta-lactam resistant nonfermenting rods (*20*). Furthermore, septic shock occurs in higher rates during gram-positive bacteremia than gram-negative bacteremia (*21*). It has been shown that for several types of spinal surgery, vancomycin powder treatment significantly reduced the rate of infection (*12, 18, 22*). Although some local outbreaks of vancomycin-resistant enterococci have occurred, they are exceptional situations (*23*). It was shown in a rat model that compared to debridement and irrigation alone, addition of vancomycin powder significantly decreased bacterial presence in contaminated open fractures (*24*). Topically applied vancomycin powder was shown to decrease the likelihood of SSIs in patients with diabetes mellitus, a subgroup with an increased likelihood of contracting SSIs, by 73% (*19*).

Vancomycin is often the last line of defense against strains of staphylococcal and streptococcal bacteria that are resistant to beta-lactam antibiotics (*25*). Vancomycin targets nascent peptidoglycan cell walls of gram-positive bacteria by interfering with cross-linking of peptide chains (*26*). Weak points in bacterial cell walls occur following the binding of vancomycin to C-

terminal D-Alanine–D-Alanine (D-Ala-A-Ala) peptides of polymeric lipid-PP-disaccharide-pentapeptides leaving targeted bacterial cells susceptible to lysis under osmotic variations (**Figure 1**) (*26*). The peptide backbone of vancomycin forms hydrogen bonds along this D-Ala–D-Ala sequence that extend from the N-acetylmuramic acid (NAM) sequence of bacterial cell walls forming a cap (*27*). Vancomycin is an asymmetric dimer that allows for the docking of two D-Ala–D-Ala peptides in opposite directions (*27*). Through natural selection after widespread use of vancomycin, vancomycin-resistant bacteria have emerged that have D-Ala–D-lactate sequences in place of D-Ala–D-Ala terminal peptides, which reduce vancomycin's binding affinity because a hydrogen bond that was once formed between a carbonyl oxygen of vancomycin and a nitrogen atom of the bacteria can no longer form as the amide of the bacteria has been replaced by an oxygen (*28*).

Similar in structure to vancomycin, the lipopeptide antibiotic daptomycin exhibits potent bactericidal activity against most clinically important gram-positive pathogens, including MRSA, vancomycin-resistant *enterococci*, coagulase-negative *staphylococci*, and penicillin-resistant *Streptococcus pneumoniae (29-31)*. Daptomycin, found in the soil bacterium *Streptomyces roseosporus*, is able to act on these resistant bacteria through a unique, calcium-dependent association with lipid membranes within these bacteria (*32*). Following interactions of daptomycin with these lipid membranes, membrane leakage occurs leading to cell death and subsequently membrane depolarization (*32*). Although antimicrobial resistance is still considered rare for daptomycin, widespread use of vancomycin and the resultant selection for bacterial cell wall thickening may cause decreased daptomycin susceptibility within some bacterial species (*33*). However, daptomycin is a safe and well-tolerated option for treating many complicated infections (*33*).

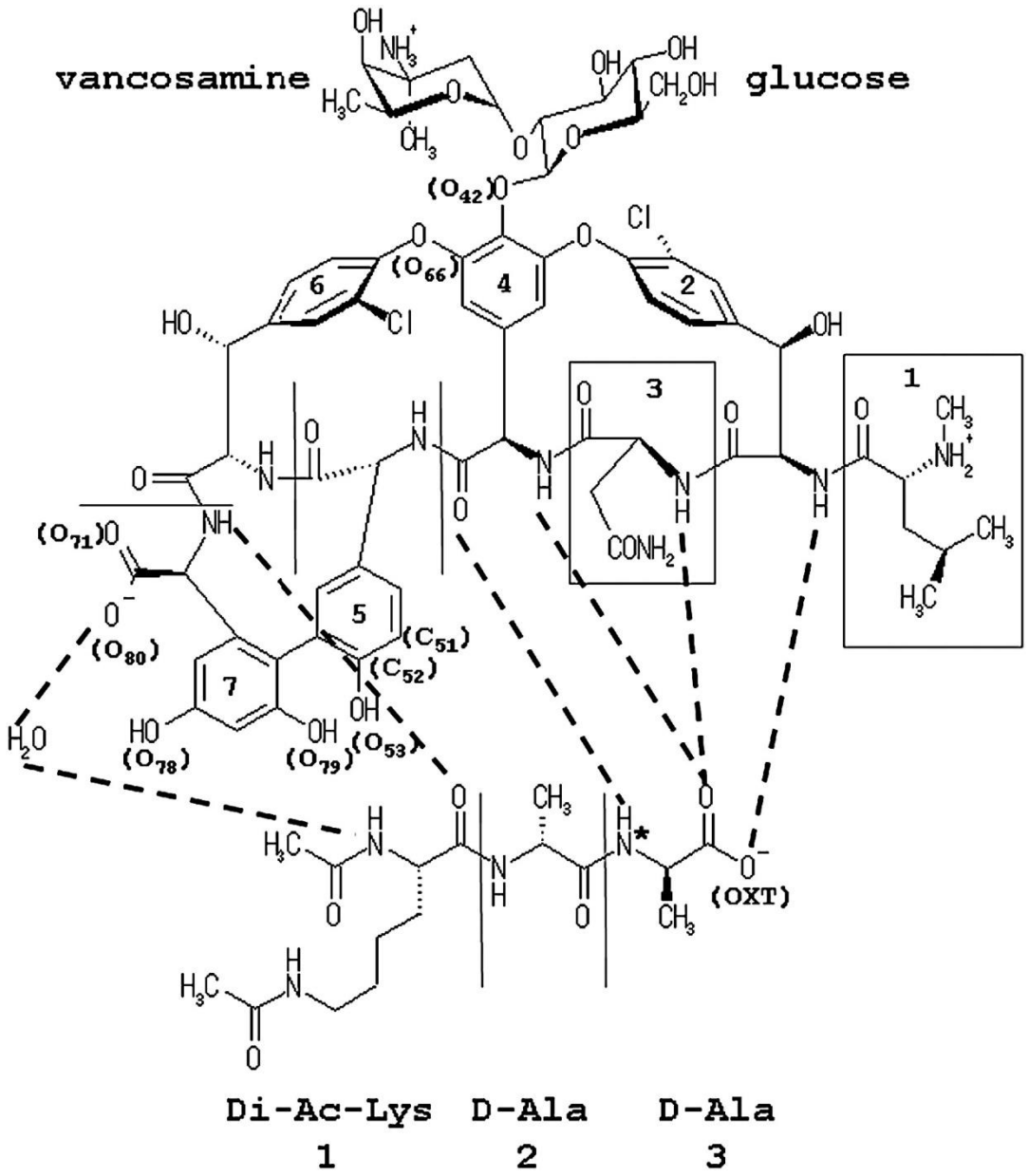

Figure 1. Chemical Structure of Vancomycin and Binding Scheme between Vancomycin and DALAA, a Cell-Wall Precursor Analogue (*27*). Amino acid residues of vancomycin and DALAA are numbered. The fourth residue of vancomycin is bound to a disaccharide of glucose and vancosamine. The hydrogen bonds that exist between vancomycin and DALAA are depicted as dotted gray lines.

Additionally, cephalosporins are a class of β-lactam antibiotics that destroy bacterial cell wall integrity through disruption of peptidoglycan synthesis (*34*). The first generation of cephalosporins targets gram-positive bacteria specifically (*34*). However, each subsequent generation exhibits increased activity against gram-negative bacteria and typically slightly less selection for gram-positive bacteria (*35*). Third generation cephalosporins exhibit broad-spectrum activity against gram-negative bacteria including *H. influenzae*, *E. coli*, and *K. pneumoniae (35-36)*. Of these third generation cephalosporins, ceftazidime is highly resistant to β-lactamases and exhibits additional activity against *Pseudomonas aeruginosa.* Ceftibuten is also highly resistant to β-lactamases and exhibits additional activity against *streptococci (37-38)*. Although no one cephalosporin is effective in treating every infection, the broad-spectrum activity of third-generation cephalosporins allows them to function effectively as the sole treatment for infections following various surgeries (*35-36, 39-40*).

Gelatin as a Hemostatic Agent during Antimicrobial Prophylaxis

Alongside antibiotics, hemostatic agents are considered almost mandatory after surgery. Several local agents are currently used including bone wax, gelatin, and fibrin glue (*41*). Gelatin began replacing clips, electrocoagulation, and ligature to obtain hemostasis in the 1940's and has been widely used ever since due to its biodegradability and biocompatibility (*42*). Although fibrin glue is still used to prevent air leakage or to stop bleeding in certain surgical cases, gelatin has a lower cost, simpler preparation, and higher adhesive strength (*43*). It was shown that cross-linked gelatin exhibited bonding strength almost three times higher than that of fibrin glue and could maintain composition under twice the water pressure through a needle hole (*43*). Cross-linked

gelatinous gel was further shown to have significantly lower rates of cytotoxicity as compared to albumin glue and successfully prevents adhesion within cecum abrasion models (*43*).

Gelatin is derived from the natural polymer collagen which is the principal component of bone, skin, and connective tissue (*44*). During the process of making gelatin, proteins are extracted from skin and bone of typically bovine or porcine sources by acid (Type A gelatin) or alkaline (Type B gelatin) baths (*45*). The alkaline process of transforming collagen into gelatin deaminates glutamine into glutamic acid and asparagine into aspartic acid leading the proportion of aspartic acid and glutamic acid to be higher in gelatin type B than gelatin type A (*46*). After bath submersion, proteins are further extracted in water with increasing temperatures in the range of 55 to 100°C yielding a mixture of free α-chains, β-chains, and γ-chains (*47*). This variation in gelatin composition leads the parameters that describe the physical and chemical structures of gelatin to be average values unlike specific descriptors of monodispersed proteins (*47*).

Collagen is composed of three alpha chains that consists of continuous repetitions of Gly-X-Y amino acid sequences where X is mostly proline and Y is mostly hydroxyproline to form a triple-helix structure (*48*). The collagen molecule exists with a molecular weight of approximately 300,000 Da, a diameter of 1.5 nm, and a length of 300 nm (*47*). Collagen presents different levels of cross-linkages and solubility based on the organ from which it is derived and the age of the animal from which it is collected (*45*). Because gelatin consists mostly of denatured collagen, it has a very similar amino acid composition to collagen, but some variations in constitution and organization occur due to manufacturing processes as shown in Table 1 (*47, 49*). Despite variations in compositions and structures across gelatins, consistently high levels of cross-links in gelatin allows it to function as a dependable hemostatic agent (*44*).

Table 1: Amino acid composition of collagen and gelatin – amino acid residues per 1000 residues

Amino Acid	Collagen	Type A Gelatin	Type B Gelatin
Alanine	114	112	117
Arginine	51	49	49
Asparagine	16	16	0
Aspartic Acid	29	29	46
Glutamine	48	48	0
Glutamic Acid	25	25	72
Glycine	332	330	335
Histidine	4	4	4
4-Hydroxyproline	104	91	93
Hydroxylysine	5	6	4
Isoleucine	11	10	11
Leucine	24	24	24
Lysine	29	27	28
Methionine	6	4	4
Phenylalanine	13	14	14
Proline	115	132	124
Serine	35	35	33
Threonine	17	18	18
Tyrosine	4	3	1
Valine	22	26	22

The high content of amino acids such as glycine, proline, and hydroxyproline function to potentially accelerate the healing of soft tissue (*50*). Increased pyrrolidine content has been linked to increased thermal stability of gelatin alongside the presence of hydroxyproline in the third position of the triplet due to increased hydrogen bond formation (*47*). The strength of gelatin has been shown to be relatively consistent within the pH range of 4 – 10 (*47*). Outside this range, gelation is greatly inhibited likely due to electrostatic forces from charged side ions preventing junction zone formation (*47*). The highly hydrophilic nature of gelatin allows for drug absorption in the form of a hydrogel and controlled drug release through a degradation or diffusion-controlled mechanism (*51*).

Existing Antibiotic-Infused Medical Devices

During post-operative procedures, surgeons may sprinkle topical vancomycin on wounds and cover it with a gelatin-based hemostatic agent. The current push for antibiotic-eluting devices to proactively prevent infections naturally translates to hemostatic agents (*52*). For example, researchers from Tel-Aviv University utilized carbodiimide to cross-link gelatin and alginate to create a bioadhesive that can be loaded with ceftazidime (*53*). The sponges were shown to maintain bondage to porcine skin for five hours in humid environments, have low cytotoxicity in rat models, and elute the loaded ceftazidime over the period of a day. Altogether, these results suggest that proper selection of adhesive components allows for biocompatible antibiotic-eluting tissue adhesives with the necessary physical properties for tailoring hemostatic agents for various post-operative procedures.

Researchers from The Hammond Lab at the Massachusetts Institute of Technology analyzed the release of gelatin from gelatin sponges to be used to absorb excess blood during invasive surgeries (*54*). Their previous development of hydrolytically degradable multilayer films allowed for the controlled release of vancomycin (*55*). To analyze vancomycin release profiles from the gelatinous sponges, slices of the sponge were soaked in phosphate-buffered saline solution (PBS) and at increasing time intervals the PBS was removed, collected, and replaced (*54*). These PBS samples were then analyzed through high performance liquid chromatography (HPLC) to determine vancomycin concentration (*54*). Furthermore, the efficacy of the antibacterial sponges was assessed through a modified Kirby–Bauer test on *S. aureus* coated plates (*54*). Ultimately, their methods increased the therapeutic properties of gelatin sponges by adding antimicrobial characteristics while increasing absorption capabilities.

From researchers at Zhongshan Hospital, gelatin sponges containing varying concentrations of b-tricalcium phosphate ceramic (β-TCP) were developed to function as a vancomycin sustained-release system (*56*). Through the use of scanning electron microscopy to identify the morphology of the composite scaffolds, Fourier transform infrared spectroscopy to investigate possible chemical interactions between gelatin and vancomycin, and *in vivo* drug release studies showed that the developed scaffolds allowed for appropriate local distribution of vancomycin over an extended duration of time (*56*). This procedure offers promise as controlled release system of antibiotics in the treatment of chronic osteomyelitis.

Also, many successful surgeries have incorporated antibiotic-infused bone cement during bone replacement surgeries (*57-58*). Through an open clinical trial, twenty patients having undergone hip prosthesis implantation that required revision due to periprosthetic infection were treated with antibiotic-loaded bone cement and the concentrations of gentamicin and vancomycin

in urine, plasma, and wound exudate were determined through quantitative liquid chromatography analysis to evaluate the systemic bioavailability of antibiotics derived from bone cement (*59*). Quantifiable amounts of both gentamicin and vancomycin were excreted within the first ten days after surgery and no reinfection occurred up to seven months after surgery (*59*). Plasma concentrations stayed well below toxic levels and did not result in a critical systemic concentration while exhibiting slow absorption rates (*59*). Overall, treatment with bone cement was shown to be efficacious and well tolerated for all patients (*59*).

*Other Uses and Mechanics of Gelatin-Based Peptide Bond Conjugation*s

In addition to bioadhesive applications, gelatin conjugates have been studied as anti-cancer agents. The Ofner lab has developed various protocols for conjugating doxorubicin and methotrexate to gelatin (*60-63*). Utilizing 1-ethyl-3-(diaminopropyl)carbodiimide HCl (EDC) as a carboxyl activating agent in peptide bond formation, methotrexate was successfully conjugated to gelatin of various molecular weights (*61*). Gelatin-methotrexate conjugates of specified molecular weight were later obtained through fractionation of gelatin with a cross-linked dextran gel column and blocking of gelatin amine sites through citraconic anhydride (*60*). With the use of glycylglycine benzyl ester p-toluenesulfonate as an organic intermediate in peptide formation, doxorubicin was able to be conjugated to gelatin (*63*). These examples exemplify the simplicity and efficiency of peptide bond formation in direct conjugation of small molecules to gelatin.

During peptide bond formation, a carboxylic acid group and an amine group undergo a condensation reaction forming an amide and releasing a molecule of water (*64*). Although the resultant peptide bond is normally depicted as a single bond, it exhibits a partial double bond

character causing the peptide unit to be a rigid and planar structure with rotation restricted around the peptide bond (*65*). This double bond nature further stabilizes the bond. For peptide bonds consisting of natural amino acids and not preceding a proline residue, the trans-form of the bond is approximately one thousand times more stable than the cis-form causing a substantial prevalence of the trans-form in peptide bonds (*66*).

To facilitate expedient peptide bond formation, carboxyl group activation is often utilized. Carboxylic acid activation replaces the hydroxyl moiety of the carboxyl group with a more electronegative group, such as a chloride, imidazolide, or anhydride, to draw electronic density from the carbonyl to increase carbonyl reactivity with amine groups (*67*). Coupling reagents, such as carbodiimide derivatives including 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and dicyclohexylcarbodiimide (DCC) in addition to carbonyldiimidazole (CDI) and its derivatives, are zero-length cross-linkers. This family of cross-linkers lead to the direct conjugation of carboxylate groups to primary amine groups without incorporation of the cross-linker components into the final amide bond to further expediate peptide bond formation (*64*).

Carbodiimides are efficient in conjugation of two protein molecules, a peptide and a protein, an oligonucleotide and a protein, a biomolecule and a particle, or any combination of these with small molecules (*68-70*). Typically, water-soluble carbodiimides, such as EDC, are used in bioconjugations because most biomacromolecules are soluble in aqueous buffer solutions while water-insoluble carbodiimides, such as DCC, are frequently used in peptide synthesis and other conjugations involving molecules soluble only in organic solvents (*68, 70*). EDC allows for direct addition to a reaction without prior organic solvent dissolution and both EDC and its by-product, isourea, are water-soluble and may be removed from the reaction easily by dialysis or gel filtration (*71*). EDC reacts with a carboxylate group to form an active ester leaving group (*69*). This reactive

complex can be slow to react with amines and can hydrolyze in aqueous solutions, having a rate constant measured in seconds (*72*). This can be a problem when the target molecule is in low concentration compared to water, as in the case of protein molecules (*69*). EDC may be used with N-hydroxysulfosuccinimide (NHS) to increase the stability and solubility of the active intermediate, which ultimately reacts with the attacking amine (*71-72*). Forming a sulfo-NHS ester intermediate from the reaction of NHS with the EDC active-ester complex greatly increases the success of resultant amide bond formation (*72*) .

CDI contains two acylimidazole leaving groups and functions as a highly active carbonylating agent that creates either zero-length amide bonds or one-carbon-length N-alkyl carbamate linkages between cross-linked carboxylic acids and nucleophiles (*73*). CDI reacts with carboxylic acid groups to form N-acylimidazoles which exhibit high reactivity (*74*). The formation of the active intermediate occurs in excellent yield due to the liberation of carbon dioxide and imidazole which function as a driving force of the reaction (*74*). This activated carboxylate readily reacts with amines to form amide bonds or with hydroxyl groups to form ester linkages (*74*).

Carbodiimide Catalyzed Reactions in Gelatin and Gelatin Degradation

Nakajima and Ikada suggested that EDC catalyzed conjugations and cross-linking reactions produce an amide bond between carboxyl and amino moieties through carboxylic anhydride mechanism recognized to occur extensively under aqueous conditions (**Figure 2**) (*75*). During the first step, EDC is protonated and forms a carbocation which leads to attack by ionized carboxyl groups forming an O-acylisourea intermediate (*75*). Then, based on concentrations and pH levels, four differing reactions may proceed. The first possible reaction

occurs in the absence of a nucleophile with water, which leads to production of urea and the regeneration of the carboxyl group (*75*). The second possible reaction produces an amide bond and the release of a urea side product through a slower and less extensive reaction that takes two steps (*75*). The third possible reaction forms an anhydride and then undergoes an extensive reaction with an amino group that results in the formation of an amide bond and a urea side product (*75*). The fourth reaction involves an O → N rearrangement to form an N-acylisourea that occurs under excess carbodiimide and neutral and mildly acidic conditions (*75*).

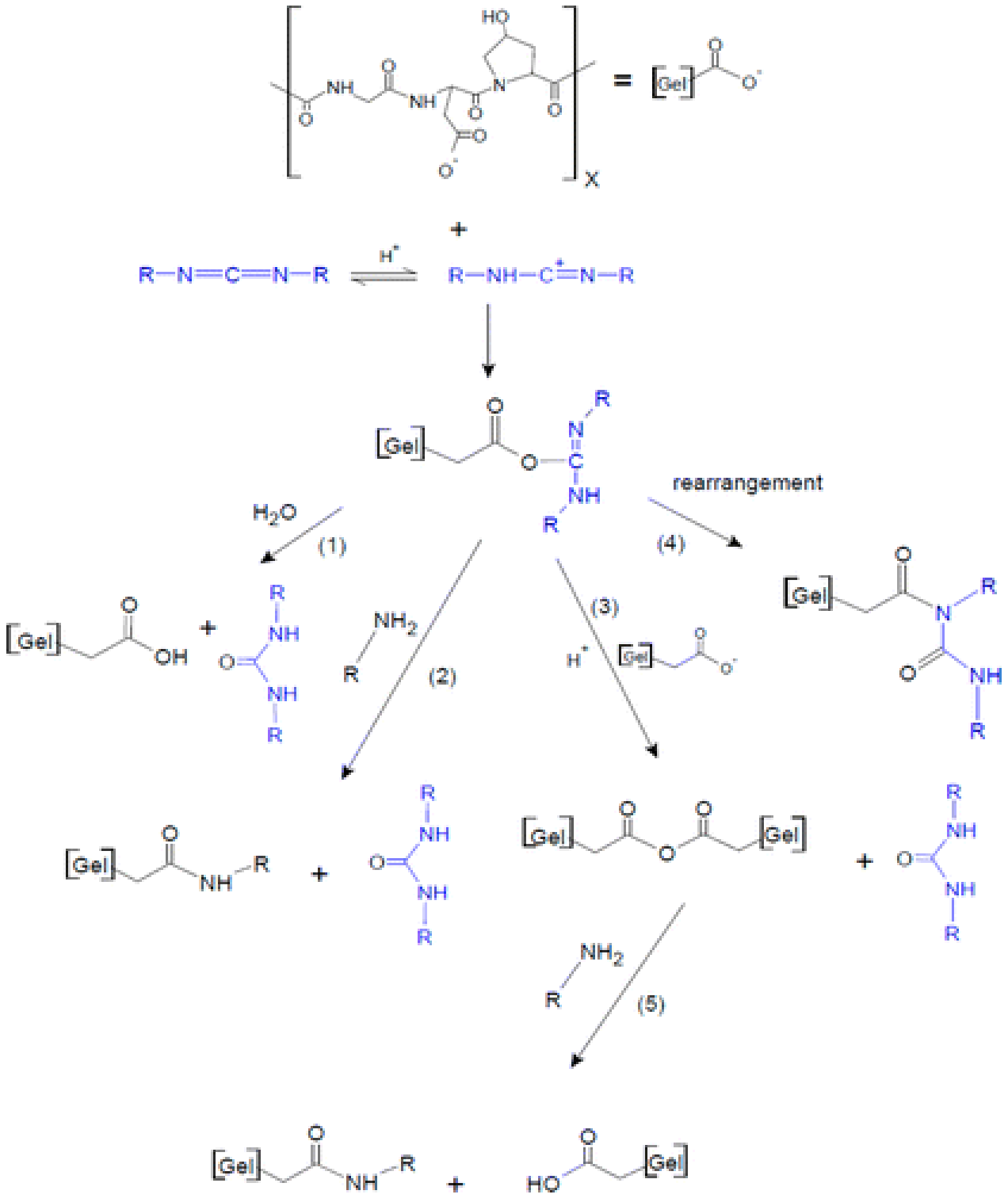

Figure 2: EDC Reactions with Gelatin after Formation of the O-Acylisourea Intermediate. (1) Reaction with water, (2) Slow reaction with amino groups, (3) Reaction with a nearby carboxyl group, (4) Formation of N-acylisourea, and (5) Fast reaction of anhydride with amino group (*75*).

EDC-induced degradation of gelatin has been shown to occur under certain conditions (*62*). Under the presence of excess EDC at a pH of 7.0 and temperatures above 25°C, substantial degradation of gelatin was observed (*62*). Moderate degradation was shown to occur at a pH 8.5 with excess of EDC (*62*). Gelatin molecules within solution have an open and random coil conformation that is most likely flexible enough to allow for close proximity of N-acylisourea imino groups relative to peptide nitrogens. This occurs at almost all intrachain amino acid residues that contain carboxyl groups facilitating reactions at intrachain sites and increasing rates of gelatin degradation (**Figure 3**) (*62*).

The Khorana mechanism is originally described with a glycylglycine benzyl derivative (**Figure 3a**) (*62*). Corresponding steps (**Figure 3b**) show gelatin peptide bond cleavage along the backbone of the molecular chain (*62*). In this mechanism, carbodiimides react with a carboxyl group to first form an O-acylisourea activated species (*62*). Then, the O-acylisourea rearranges into a N-acylisourea species, followed by a nucleophilic addition of a peptide nitrogen to the N-acylisourea to form an intermediate ring (*62*). Under the presence of a hydroxyl ion at neutral or higher pH, the ring breaks, splitting the EDC adduct and cleaving the peptide bond adjacent to the ring yielding a peptide chain that ends in a carboxyl group and a peptide chain ending with a split EDC adduct (*62*).

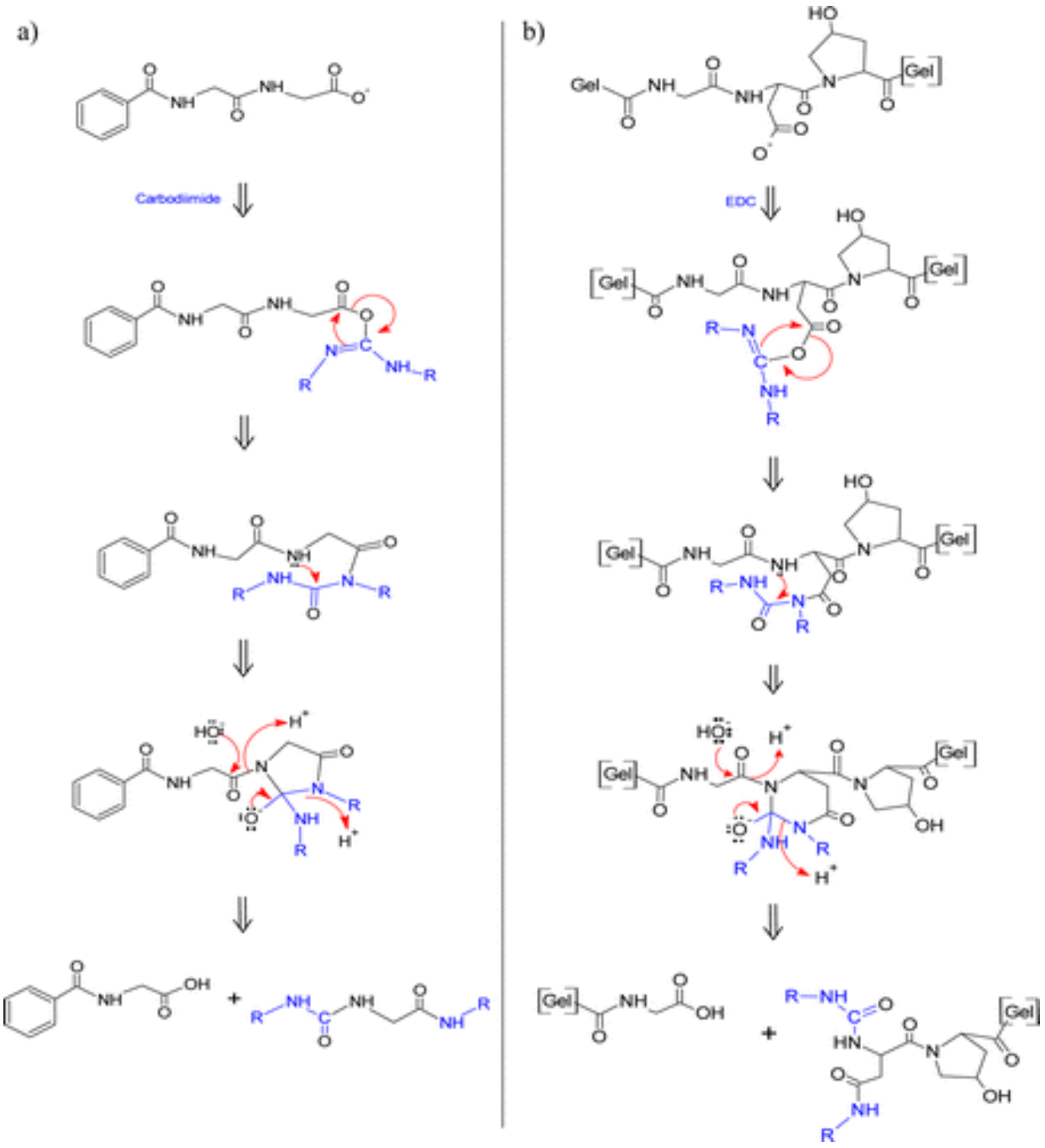

Figure 3: Proposed Mechanism of EDC-Induced Degradation of Gelatin as an Extended Khorana Mechanism. (A) Khorana mechanism for peptide bond cleavage of terminal amino acid. (B) Extended Khorana mechanism for peptide bond cleavage of an intrachain amino acid in gelatin (*62*).

Methods for Analysis of Conjugation

To allow for a quantitative confirmation of successful conjugation, vancomycin-gelatin conjugates can be purified through size exclusion chromatography and undergo mass determination by mass spectroscopy. During mass spectroscopy analysis, a sample is ionized causing some to break into charged fragments. These ions are then accelerated and subjected to an electric or magnetic field separating them according to their mass-to-charge ratio (*76*). The ions are then detected by an electron multiplier and spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio are displayed (*76*). This spectra can be used to determine the masses of molecules and elucidate their chemical structures through characteristic fragmentation patterns or correlation of known masses of chemical compounds to displayed masses within the spectra (*76*).

In order to determine the effectiveness of antibiotic-eluting devices, release profile analysis can be performed. The structure under analysis is submerged within solution and over incremental time periods the liquid is siphoned off, collected, and replaced. The presence of an antibiotic in the eluent and the concentrations of an antibiotic within the eluent can be determined through ultraviolet-visible (UV-Vis) spectroscopy and liquid chromatography-mass spectrometry (LC-MS). To determine concentrations of an antibiotic within a sample through UV-Vis spectroscopy, absorbances at a characteristic wavelength (280 nm for vancomycin) can be compared to a standard curve of absorbances of known concentrations of the antibiotic within the same buffer solution. An LC-MS determines concentrations within a solution by separating the components within the solution through liquid chromatography and then identifying the structure of each component within the solution through mass spectroscopy (*77*). Structural integrity of the eluent can be confirmed through infrared (IR) spectroscopy. IR spectroscopy utilizes photons from the infrared

range to create unique spectra for molecules based on the interactions between the sample and the photons (*78*).

Summation of Thesis

To limit the prevalence of SSIs and their negative implications on the healthcare industry and society, antimicrobial prophylaxis procedures need to be optimized. Incorporation of antibiotics into hemostatic agents should increase antimicrobial presence within incision sites and improve the efficacy of antimicrobial prophylaxis. In addition, local application can reduce unnecessary systemic administration. This prevents the death of healthy bacteria within patients and limits selection for antibiotic-resistant bacteria through reduction of the application area of the antibiotics. The current investigation developed procedures for the conjugation of small molecules to gelatin through peptide bond formation to produce antibiotic-infused hemostatic agents. This allows for the conjugation of antibiotics to gelatin and the trapping of these antibiotics within cross-linked gelatin. The glycopeptide vancomycin was the primary antibiotic analyzed throughout this study. Additionally, the lipopeptide daptomycin and the two cephalosporins ceftazidime and ceftibuten were incorporated into gelatin-based hemostatic agents.

There are two possible forms of conjugation between gelatin and vancomycin: the conjugation of a carboxylic acid on vancomycin to an amine on gelatin (**Figure 4a**) and the conjugation of an amine on vancomycin to a carboxylic acid on gelatin (**Figure 4b**). Cross-linking of gelatin to entrap vancomycin within a gelatin cage is performed through activation of gelatin carboxylic acids by the zero-length cross-linking agent EDC (**Figure 5**). Additionally, these methodologies can be extended to daptomycin, ceftazidime, and ceftibuten (**Figure 6**).

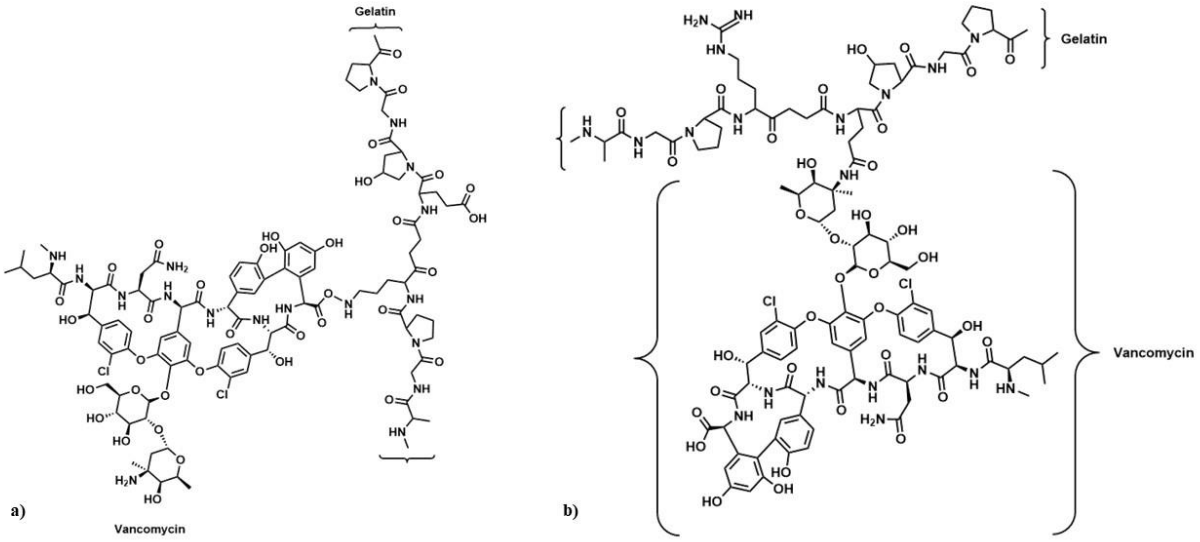

Figure 4. Possible Conjugations of Vancomycin with Gelatin. (A) Conjugation of a carboxylic acid on vancomycin to an amine on gelatin. (B) Conjugation of an amine on vancomycin to a carboxylic acid on gelatin.

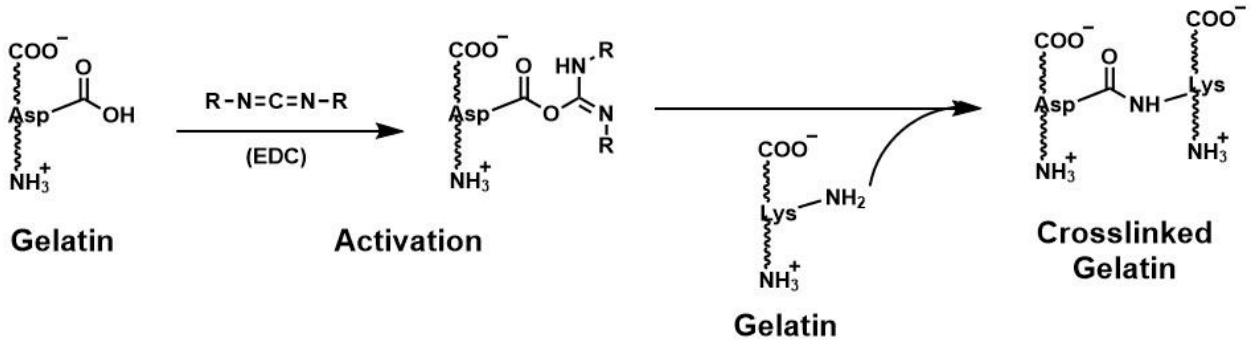

Figure 5. Schematic of Gelatin Cross-linking through EDC Activation.

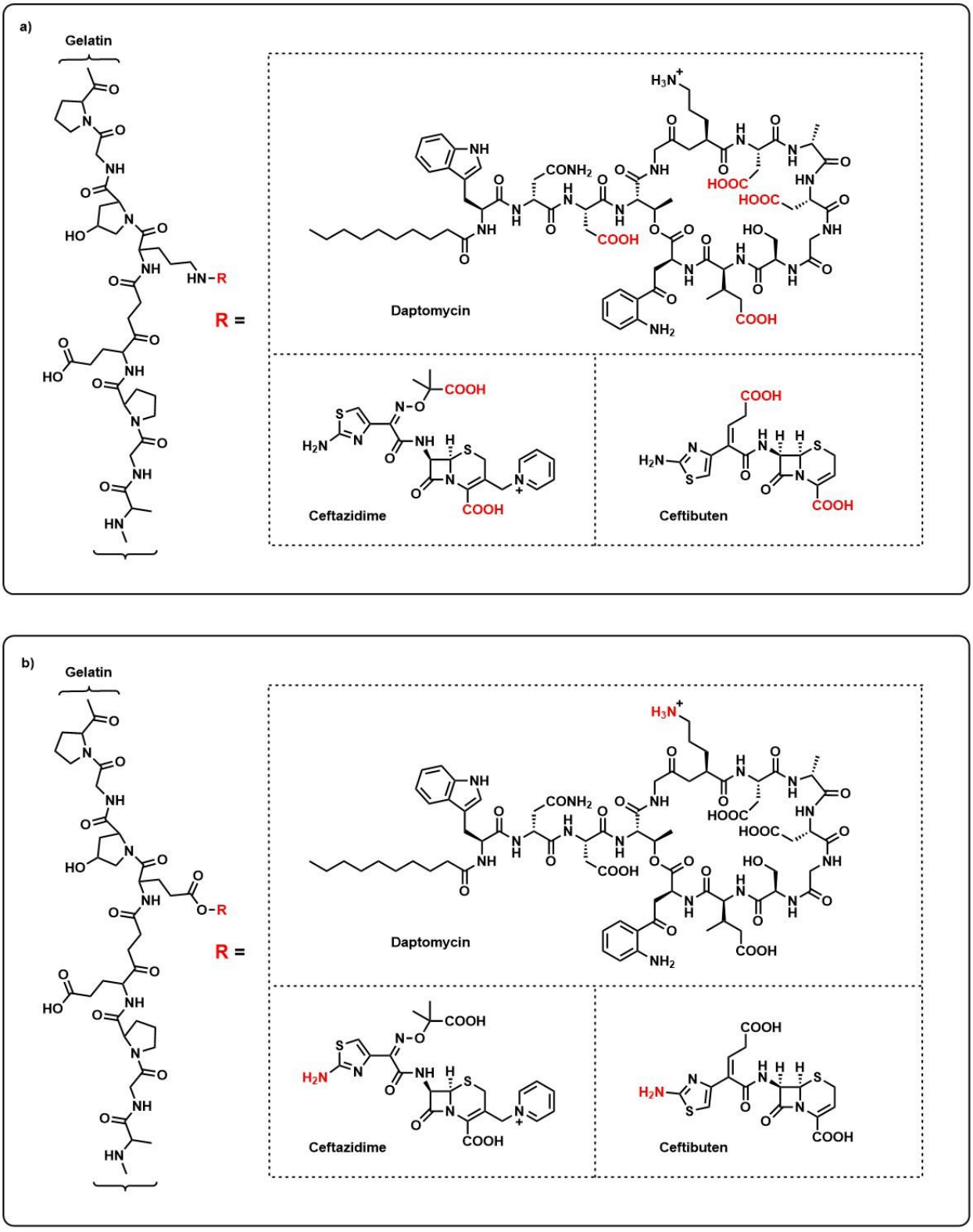

Figure 6. Possible Conjugations of Additional Antibiotics with Gelatin. (A) Conjugation of a carboxylic acid on the antibiotics to an amine on gelatin. (B) Conjugation of an amine on the antibiotics to a carboxylic acid on gelatin.

Previous methods of conjugations with gelatin were initially modified for conjugation with vancomycin. Vancomycin release profiles were analyzed from samples of various reactant ratios to optimize reaction conditions. Antibacterial activity and structural integrity of eluted vancomycin was confirmed. The biocompatibility and structural makeup of the conjugations were additionally determined. Furthermore, the developed protocol was applied to conjugations of gelatin to daptomycin, ceftazidime, and ceftibuten. The produced hemostatic agents are currently under further development for use in antimicrobial prophylaxis procedures following surgery.

Materials and Methods

Materials

Gelatin type B, 2-(N-morpholino)ethanesulfonic acid (MES), vancomycin, daptomycin, ceftazidime, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and absolute ethanol were purchased from Fischer Chemical. Ceftibuten and NaCl were purchased from Sigma-Aldrich (St. Louis, MO). Tryptic soy agar and broth were purchased Difco Laboratories (Sparks, MD). HEK-293 fibroblasts and *Staphylococcus aureus* 25923 were obtained from ATCC (Manassas, VA).

Synthesis of Antibiotic-Eluting Hemostatic Agents

Gelatin was prepared as a 20 mg/mL stock solution within 0.05 M MES buffer pH 5.0 and stirred at 50°C until soluble. For the synthesis of the conjugates, 20 mM carboxyl group concentration from the gelatin stock was incubated at 22°C with either vancomycin (2.07 mM), daptomycin (1.85 mM), ceftibuten (7.31 mM), or ceftazidime (5.49 mM) under activation by EDC (50 mM) for 2 hours and shaking at 50 rpm. The product was then precipitated under ice-cold absolute ethanol, centrifuged at 6000x g, dissolved in 1.85 mM NaCl for washing, and precipitated a second round. Conjugates were then vacuum dried and incubated at 80°C to remove any residual ethanol residing within the conjugates.

Rate of Release and Structural Integrity of Antibiotics Eluted from Synthesized Hemostatic Agents

For release profile determination, conjugates were combined with 0.5 mL phosphate-buffered saline solution (PBS) to form a semisolid structure which was then immersed in 1 mL PBS. Samples were incubated at 37°C and 100% relative humidity over a period of 3 weeks. 1 mL

of PBS was removed and replaced with fresh PBS at 24, 48, 72, 96, 168, 336, and 504 hours to obtain release samples. Release samples were stored at -80°C until analyzed. Antibiotic concentrations were initially determined through UV analysis with a Thermo Scientific Genesys 10S UV-Vis. High performance liquid chromatography (Waters HPLC, 1100 series) was then used to determine antibiotic concentrations. Samples were run for 10 minutes using 70/30 PBS/methanol mobile phase, 1 mL/min flow rate with a 150 μL injection volume on a C18 reverse phase column (Supelco) coupled with UV detection (280 nm for vancomycin, 223 nm for daptomycin, 254 for ceftazidime, and 228 for ceftibuten). Peak height was then correlated with standards of known concentrations of antibiotics used within the conjugate to determine the concentration of antibiotics in the released samples. Fourier transform infrared analysis was additionally performed on released antibiotics through the use of a Nicolet Is10 infrared spectrometer to confirm structural integrity.

Bacterial Growth Inhibition and Efficacy of Released Antibiotics

Inhibition of *S. Aureus* by synthesized hemostatic agents was determined through modified Kirby-Bauer disk diffusion tests and microdilution assays. Throughout all Kirby-Bauer tests, agar plates were formed using tryptic soy agar. The plates were subsequently coated with *S. Aureus* within its exponential growth phase at a concentration of 10^8 CFU/mL. Filter papers that had been incubated with the hemostatic agents, gelatin, or pure antibiotic equivalent to 24-hour release concentrations for two hours were then immediately placed upon the plates. Zones of inhibition were measured and photographed following 16-18 hours of incubation at a temperature of 37°C. Microdilution assays were performed in triplicate using *S. Aureus* in 96-well plates and performed in serial two-fold dilutions. Bacteria grown to their exponential growth phase while diluted in

tryptic soy broth to 10^5 CFU/mL were used within the assays. Following 16 to 18-hour incubations at 37°C, optical density at 600 nm for treated and control bacteria was examined through a BioTek PowerWave XS plate reader. The normalized bacterial inhibition was calculated from Equation (1).

$$\text{Normalized bacteria inhibition} = \frac{(\text{OD600 positive control} - \text{OD600 sample})}{(\text{OD600 positive control} - \text{OD600 negative control})} \quad (1)$$

Biotoxicity Analysis of Hemostatic Agents

Conjugate biocompatibility was established by examining cell viability of HEK 293T fibroblasts upon exposure to conjugate eluent, pure vancomycin, and gelatin samples. HEK-293T cells were cultured in complete media (DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 1 mM penicillin/streptomycin at 37°C in 5% CO2). For biocompatibility analysis, cells were seeded at 10,000 cells per well in 96-well polystyrene tissue culture plates and incubated in complete media (100 uL) at 37ºC for 24 hours. Conjugates were allowed to completely elute within culture media at 37ºC for 24 hours. Gelatin (0.3 mg/mL) and pure antibiotics (0.1 mg/mL) were also incubated within culture media at 37ºC for 24 hours. All samples were filtered through 0.2 μm filters and used (100 μL) to replace the media on the growing cells. Cells cultured in untreated media were positive controls, while untreated media without cells were used as negative controls. After an 18-hour exposure to the test media, dye solution (15 μl) was administered to each well and the plate was incubated at 37°C for 3 hours within a humidified, 5% CO2 atmosphere. Following incubation, solubilization solution/stop mix (100 μl) was added to each well. A BioTek PowerWave XS plate reader was then used to detect the absorbance of the wells at 600 nm to elucidate cell viability. Cell viability was calculated from Equation (2).

$$\text{Normalized cell viability} = \frac{(\text{Abs600 sample} - \text{Abs600 negative control})}{(\text{Abs600 positive control} - \text{Abs600 negative control})} \quad (2)$$

Structural Analysis of Hemostatic Agents

Scanning electron microscopy (SEM) was performed on conjugates following drying procedures. The morphology of the conjugates was determined through the use of a JEOL JSM-6490 microscope. Conjugates were fixed in a 10% formaldehyde solution for 2 hours, dehydrated through a series of ethanol washes with increasing concentrations, and mounted on double-sided conductive carbon tape.

Results

To identify gelatin concentrations most appropriate for extended release, gelatin concentrations in the generalized vancomycin-gelatin conjugation procedure were varied from 5 mg/mL to 20 mg/mL and the release profile for vancomycin eluted from samples loaded with 2 mg/mL (**Figure 7**) and 3 mg/mL (**Figure 8**) vancomycin was determined over a three-week period.

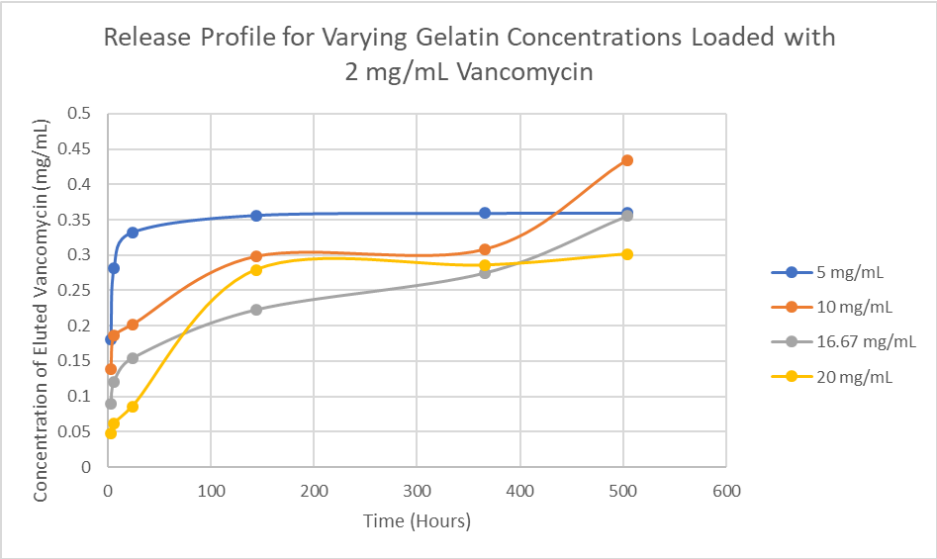

Figure 7. Release Profiles for Varying Gelatin Concentrations Loaded with 2 mg/mL Vancomycin over a Three-Week Period. Samples were analyzed through UV-Vis and collected within PBS solution.

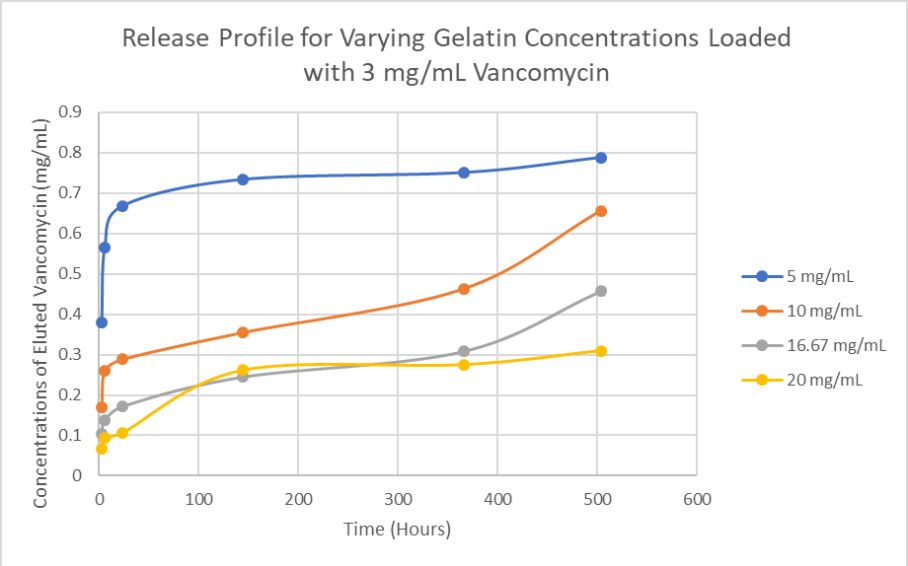

Figure 8. Release Profiles for Varying Gelatin Concentrations Loaded with 3 mg/mL Vancomycin over a Three-Week Period. Samples were analyzed through UV-Vis and collected within PBS solution.

Additionally, the release profiles for more moderate-range concentrations of gelatin were analyzed over a two-week period (**Figure 9**). The amount of EDC used within this procedure was varied and the amount of vancomycin released from samples loaded with 2 mg/mL vancomycin was determined over a two-week period (**Figure 10**).

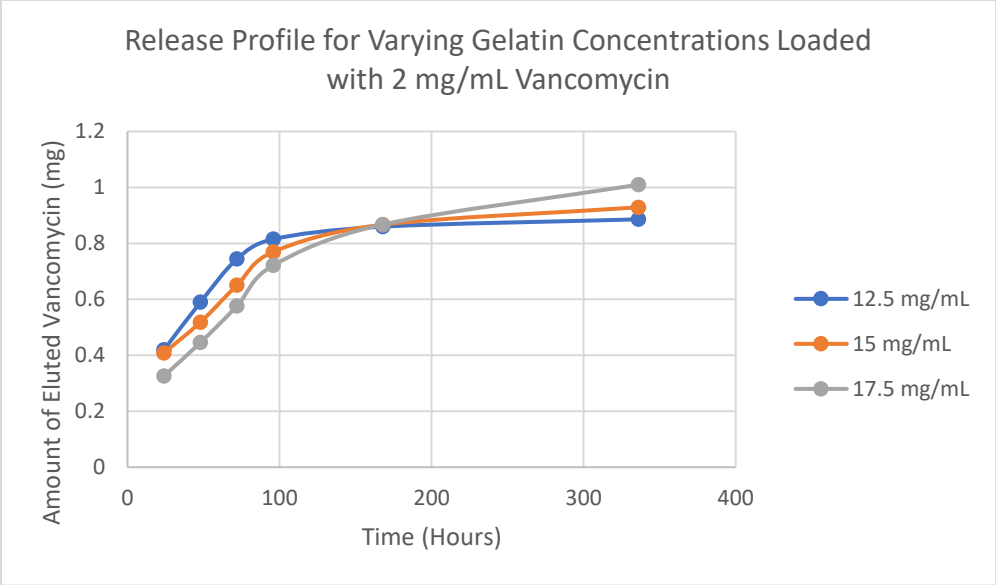

Figure 9. Release Profiles for Varying Gelatin Concentrations Loaded with 2 mg/mL Vancomycin over a Two-Week Period. Samples were analyzed through UV-Vis and collected within PBS solution.

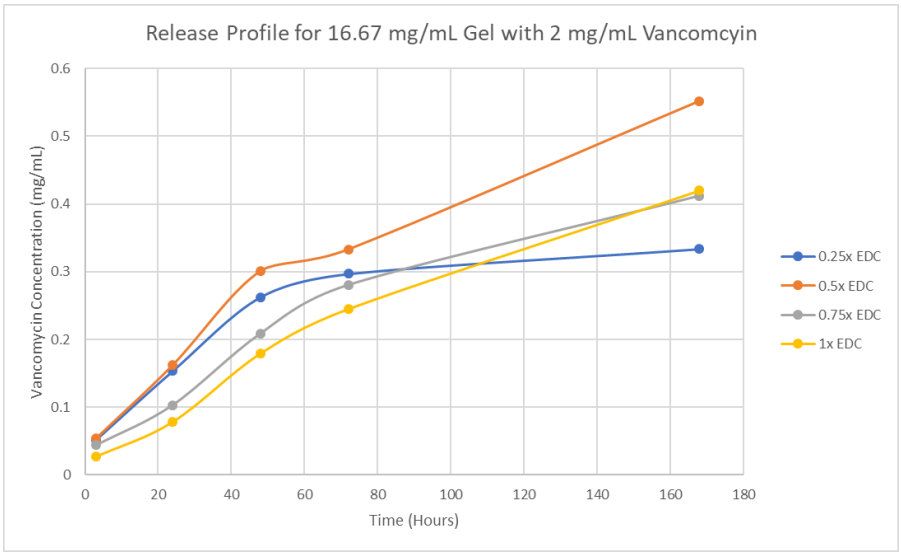

Figure 10. Release Profiles for Varying EDC Concentrations Loaded with 2 mg/mL Vancomycin over a Two-Week Period. Samples were analyzed through UV-Vis and collected within PBS solution.

Additionally, release profiles for vancomycin-gelatin conjugates over a two-week span at 37°C were determined and compared to those previously performed at 21°C (**Figure 11**). An increased rate of release was seen throughout the higher temperatures.

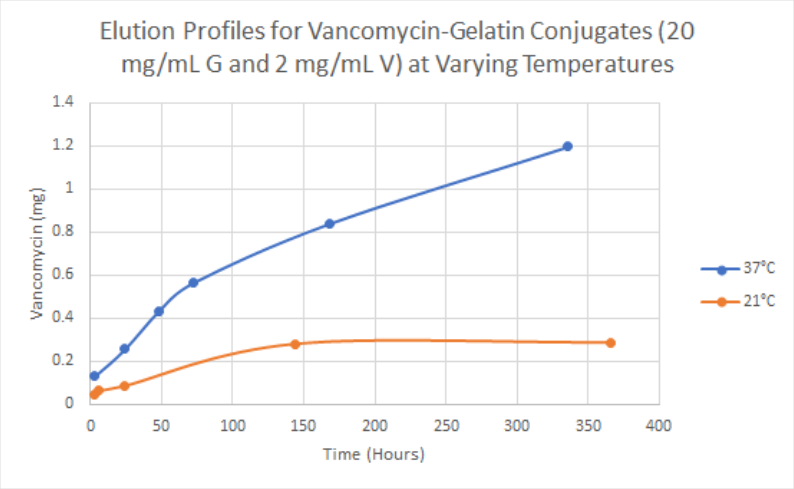

Figure 11. Release Profiles for 20 mg/mL Vancomycin-Gelatin Conjugates Loaded with 2 mg/mL Vancomycin over a Two-Week Period at Both 21°C and 37°C. Samples were analyzed through UV-Vis and collected within PBS solution.

HPLC was utilized to determine release profiles from vancomycin and daptomycin conjugates to compare release profiles at physiological temperature. Release profiles were run in triplicate over a three-week period (**Figure 12**). Vancomycin conjugates revealed a large initial burst effect with a release of 10% of the loaded vancomycin within 48 hours. Following the initial burst, release of vancomycin was sustained for over a period of 500 hours until the hemostatic agent dissolved and separation was no longer practical. Daptomycin conjugates initially showed a much slower, linear release. Akin to the vancomycin conjugates, the daptomycin conjugates released approximately 20% of the total antibiotic near 500 hours and the complex slowly collapsed following that period.

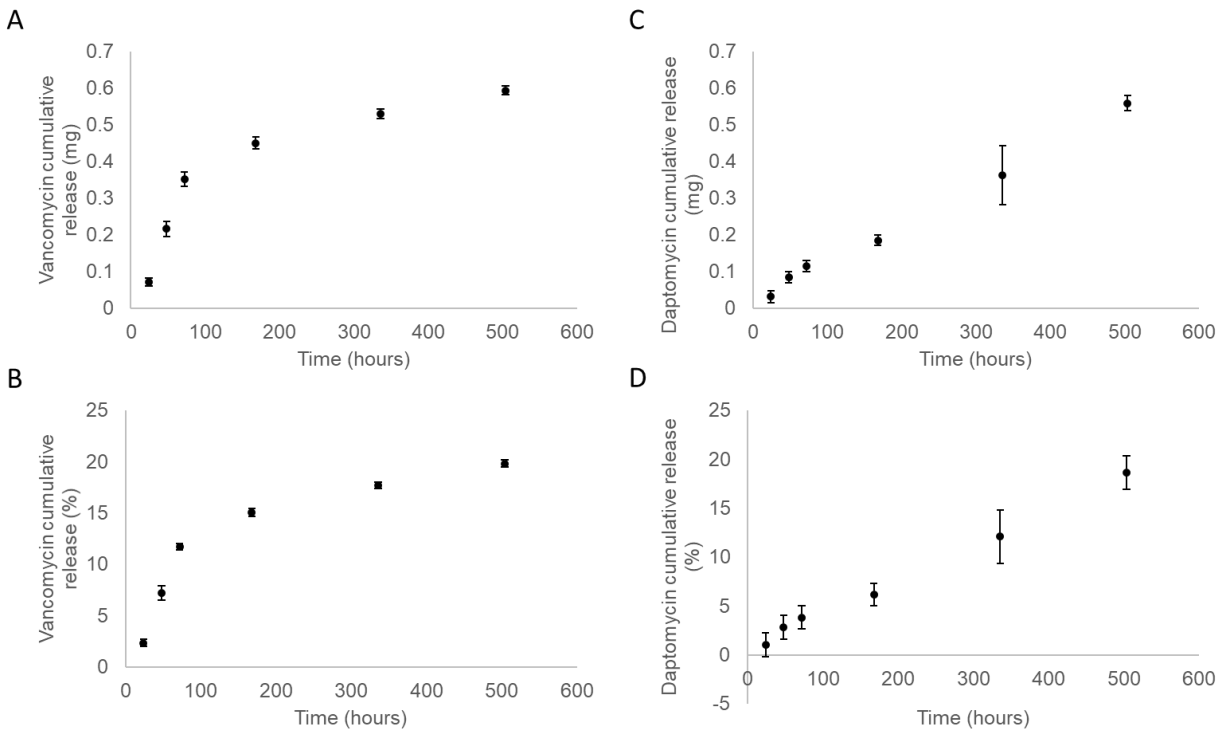

Figure 12. *In Vitro* Release of Antibiotic from Antibiotic-Eluting Hemostatic Agents. Each preparation was loaded with indicated antibiotic concentrations. (A & C) Cumulative release (mg) of vancomycin and daptomycin, respectively. (B & D) Cumulative release (%) of total antibiotic loaded in the hemostatic agent for vancomycin and daptomycin, respectively.

The structural integrity of vancomycin released from samples composed using varying EDC concentrations was evaluated through the use of IR spectroscopy to compare spectra of the conjugates to that of pure vancomycin after 72 hours at 21°C (**Figure 13**) and 2 weeks at 21°C (**Figure 13**). It was seen that at all time points analyzed that the IR spectra was not substantially changed from that of the standard.

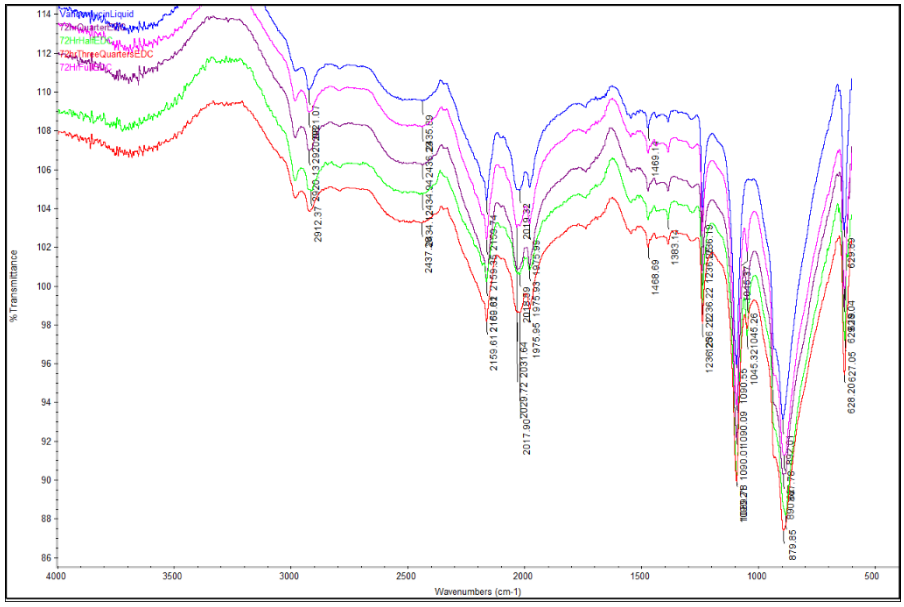

Figure 13. Infrared Spectra for 72-Hour Releases from Varying Gelatin Concentrations as Compared to Pure Vancomycin (Blue). Each sample was analyzed within PBS solution and total transmittance was compared. Samples are staggered for viewing comparisons.

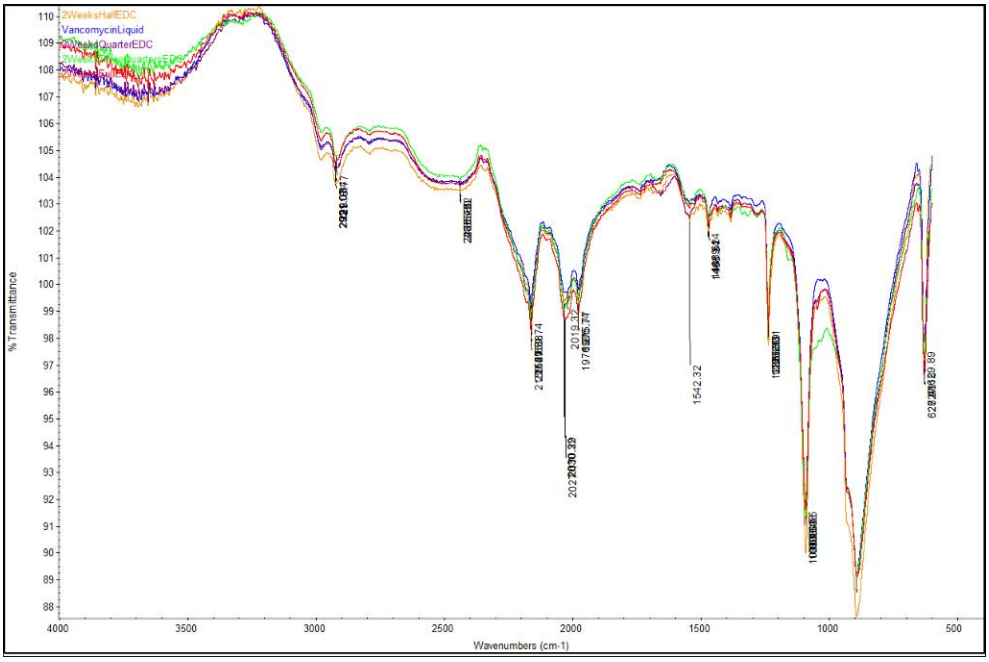

Figure 14. Infrared Spectra for 2-Week Releases from Varying Gelatin Concentrations as Compared to Pure Vancomycin (Purple). Each sample was analyzed within PBS solution and total transmittance was compared. Samples are overlaid for viewing companions.

The structural integrity of antibiotics released from vancomycin-based conjugates, along with daptomycin conjugates, over a two-week period at 37°C was analyzed and confirmed through IR spectroscopy (**Figure 15**). It was seen that the IR spectra of the conjugates was not substantially different from that of the standard.

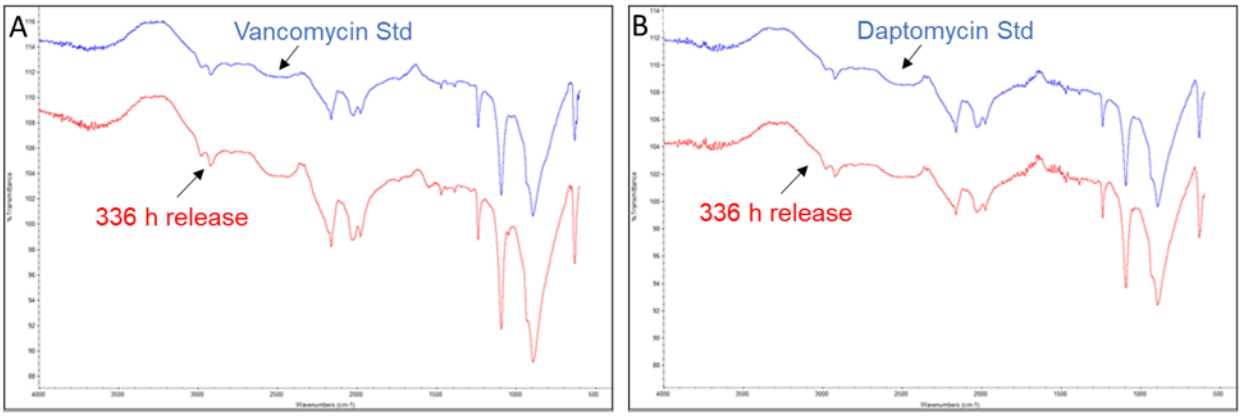

Figure 15. FTIR Spectra of Antibiotic Released from Hemostatic Agents. Pure vancomycin or daptomycin standards were compared to samples released from hemostatic agents. Pure vancomycin (A) or daptomycin (B) are both in blue and samples released from the hemostatic agents after 2 weeks are both in red.

In order to obtain structural information about the conjugates themselves, conjugates were prepared for electron microscopy and imaged at various magnifications. Conjugates incorporating daptomycin and vancomycin were directly compared to show variations in morphology (**Figure 16**).

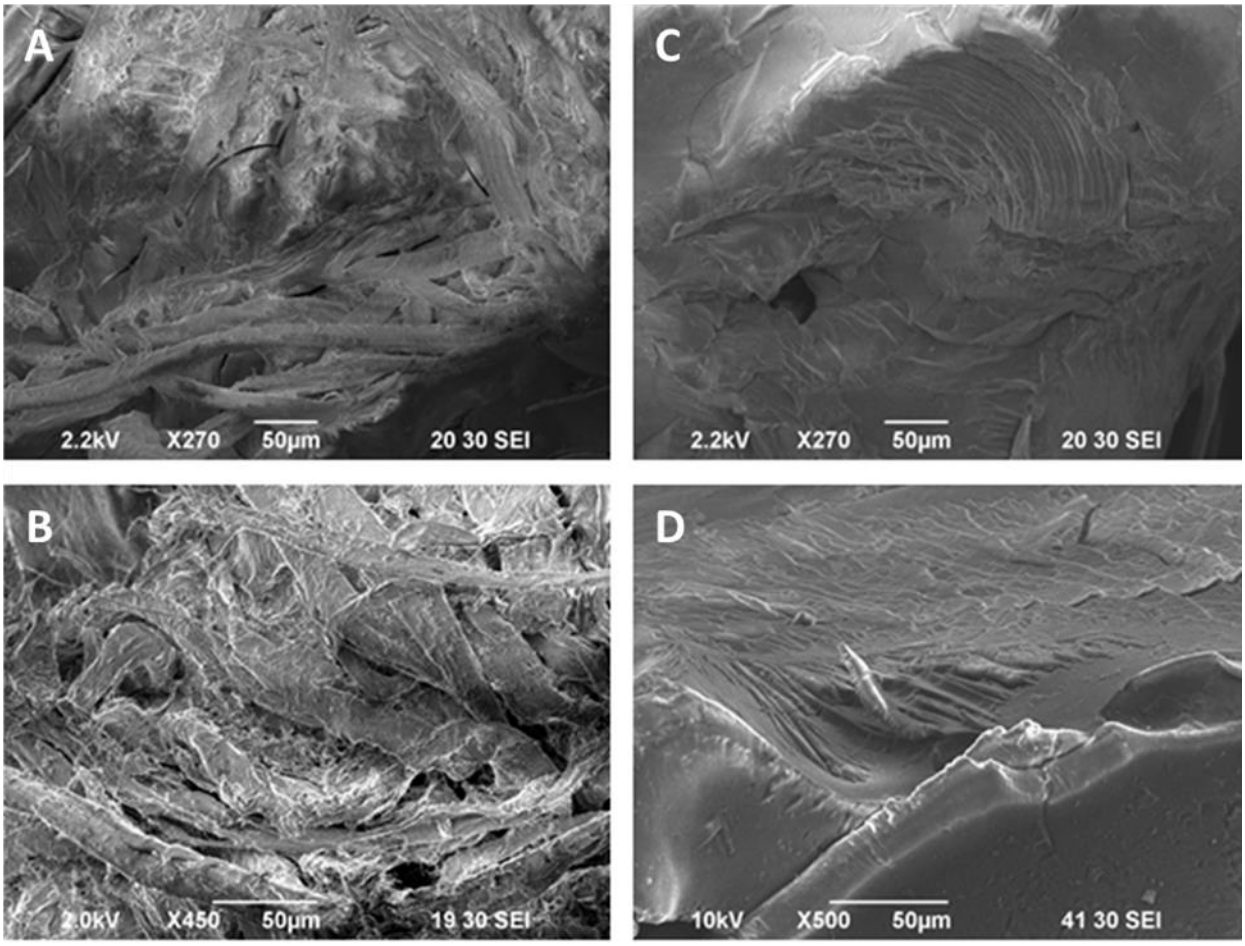

Figure 16. SEM Micrographs of Antibiotic-Releasing Hemostatic Agents. (A & B) Vancomycin conjugates at 270x and 450x magnifications, respectively. (C & D) Daptomycin conjugates at 270x and 500x magnifications, respectively.

Following confirmation of the structural integrity of vancomycin, Kirby-Bauer tests were run with vancomycin-gelatin conjugates to test for the inhibition of *S. Aureus* growth. It was seen that conjugates loaded with 2 mg/mL vancomycin and 3 mg/mL vancomycin did inhibit the growth of *S. Aureus* with a direct relationship between the amount of loaded antibiotic and the size of the zone of inhibition (**Figures 17 and 18**).

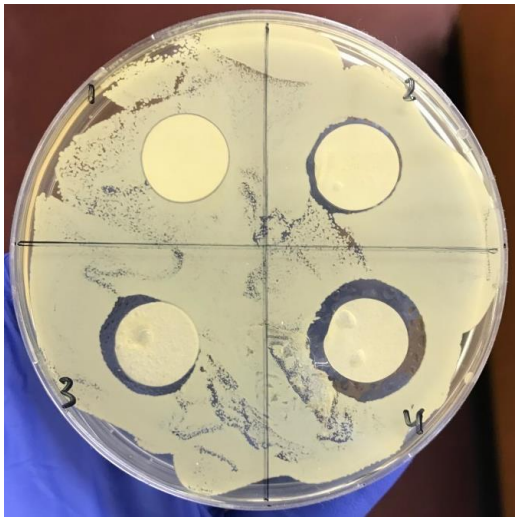

Figure 17. Kirby-Bauer Test of Inhibition using *S. Aureus*. (1) Cross-linked gelatin, (2) Conjugate produced from 16.67 mg/mL gelatin loaded with 2 mg/mL vancomycin, (3) Conjugate produced from 16.67 mg/mL gelatin loaded with 3 mg/mL vancomycin, (4) Vancomycin (0.1 mg/mL).

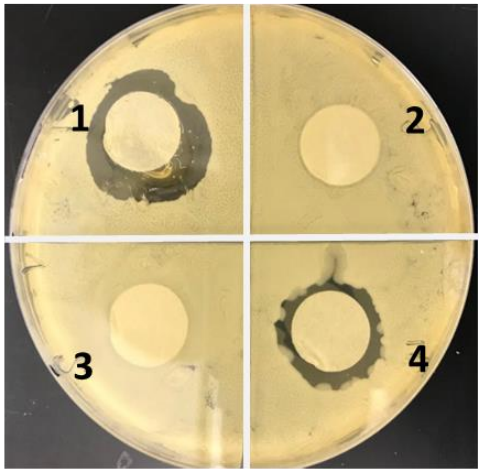

Figure 18. Kirby-Bauer Test of Inhibition using *S. Aureus*. (1) Conjugates at 20 mg/mL gelatin loaded with 3 mg/mL vancomycin, (2) Cross-linked gelatin, (3) Filter Paper, (4) Vancomycin (0.1 mg/mL).

In order to determine the efficacy of vancomycin released from antibiotic-eluting hemostatic agents, microdilution assays were performed (**Figure 19**). The therapeutic efficiency of the released vancomycin was established through monitoring the normalized density of *S.*

aureus cultured within the presence of dilutions of the released vancomycin. Efficacy remained consistent between 48-hour and 336-hour samples, further indicating structural integrity of the released vancomycin and its retained ability to sustain inhibition of bacterial growth over the course of the release. Vancomycin released from the hemostatic agent displayed an MIC between 0.5 and 2 μg/mL as previously shown in the literature for pure vancomycin (*16-19*).

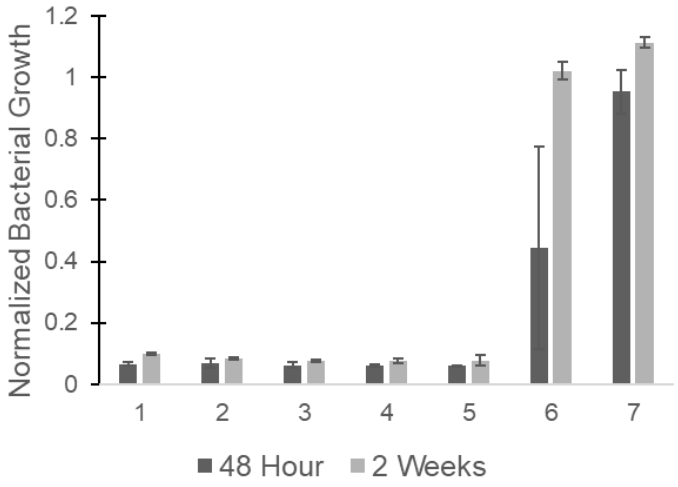

Figure 19. Bacterial Inhibition by Vancomycin Released from Hemostatic Agents. Cultured *S. aureus* was subjected to vancomycin released from either 48-hour samples or 2-week samples of antibiotic-eluting hemostatic agent. Dilution 1 contains 9.875 μg/mL for the 48-hour samples and 18.25 μg/mL vancomycin for the 2-week samples. Subsequent dilutions reflect a 50% reduction in concentration of the previous dilution.

Additionally, the cellular viability of human fibroblasts within the presence of the synthesized antibiotic-eluting hemostatic agents was determined to ensure that synthesized conjugates were biocompatible. Media incubated with prepared hemostatic agents containing released antibiotic, control concentrations of antibiotic, and cross-linked gelatin were administered to cultured cells. An MTT assay was then utilized to determine cellular viability (**Figure 20**). It

was seen that cells under exposure to the conjugates were of comparable viability to that exposed to pure antibiotics or gelatin.

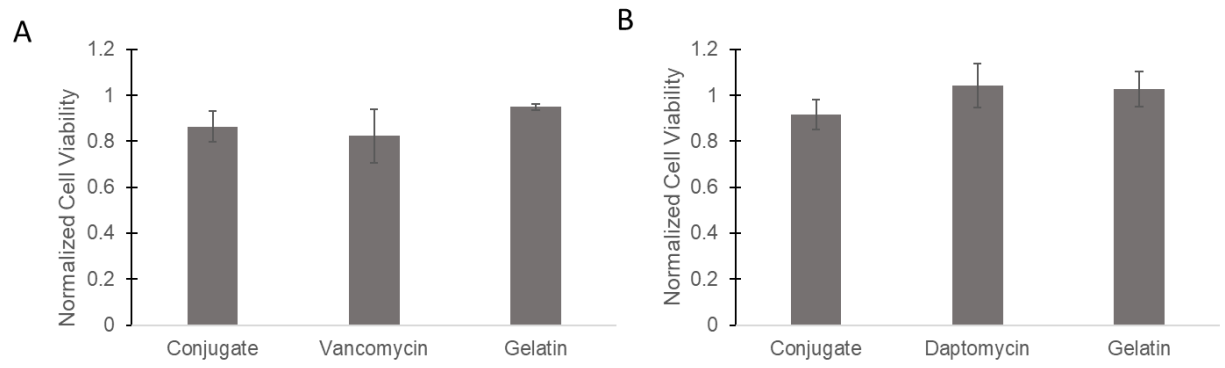

Figure 20. Normalized Cell Viability for Fibroblasts Treated with Antibiotic-Eluting Hemostatic Agents. (A) Viability of cells determined in response to released vancomycin from vancomycin-eluting hemostatic agent samples after 48 hours and controls including equivalent concentration of vancomycin and cross-linked gelatin. (B) Viability of cells determined in response to daptomycin-eluting hemostatic agent and equivalent controls.

In addition to assessment of conjugates derived from vancomycin and daptomycin, assessments of conjugates synthesized with cephalosporins were performed. Release profiles of ceftazidime-linked hemostatic agents were determined by HPLC in triplicate (**Figure 21**). Ceftazidime conjugates showed steady and nearly equal release at earlier collection points up to one week. Afterwards, slower release of ceftazidime was sustained for over a period of 500 hours until separation was no longer practical.

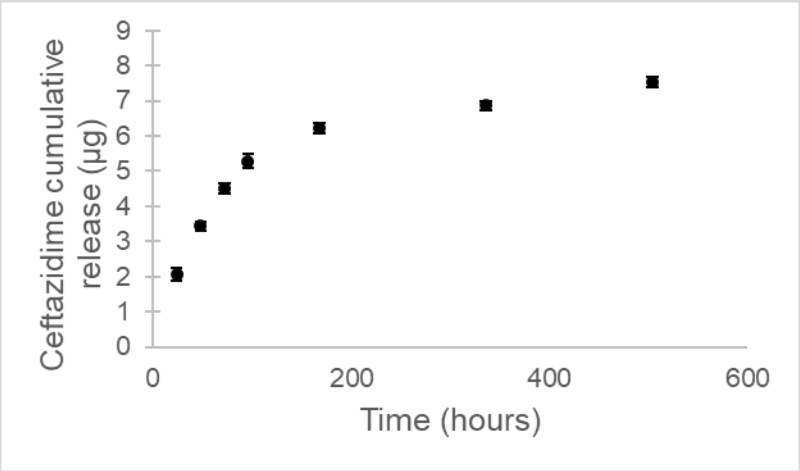

Figure 21. *In Vitro* Release of Ceftazidime from Synthesized Antibiotic-Eluting Hemostatic Agents. Each preparation was loaded with 3 mg antibiotic concentrations and cumulative release (µg) is shown.

Conjugation of cephalosporins to gelatin via the activity of EDC may cause structural changes to the antibiotic itself. As such, FTIR spectral data was obtained of the released antibiotic samples. Basic chemical stability of ceftazidime was confirmed in all released samples as seen in representative samples (**Figure 22**).

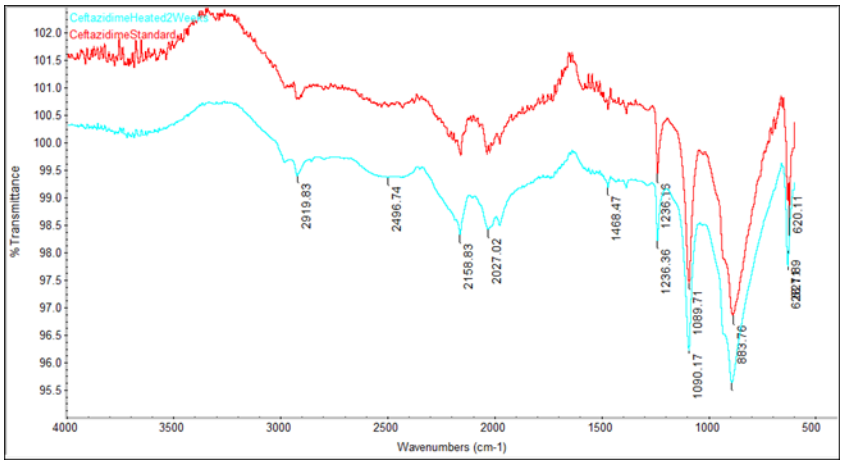

Figure 22. FTIR Spectra of Antibiotic Released from Hemostatic Agents. Pure ceftazidime standard was compared to samples released from hemostatic agents. Pure ceftazidime (red) is compared to samples released from the hemostatic agents after 2 weeks (blue).

Additionally, cell viability of human fibroblasts in the presence of the ceftazidime-eluting hemostatic agents was assessed (**Figure 23**). Media incubated with prepared hemostatic agents containing released antibiotic, control concentrations of antibiotic, and cross-linked gelatin were administered to cultured cells. An MTT assay was then utilized to determine cellular viability. Cellular viability of cells exposed to released samples of ceftazidime was seen to be lowered slightly as compared to untreated healthy cells.

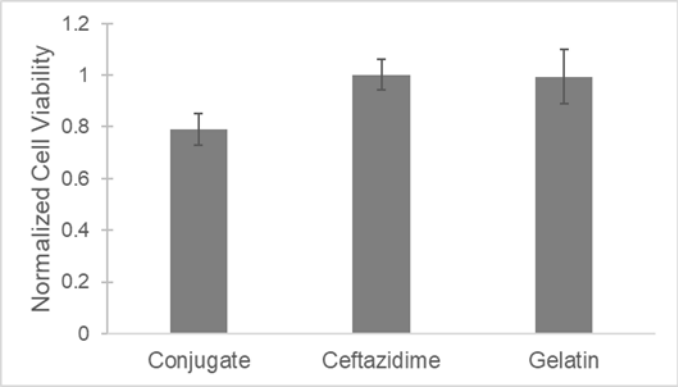

Figure 23. Normalized Cell Viability of Fibroblasts in Response to Ceftazidime-Eluting Hemostatic Agent Samples. Samples were taken from 48-hour release and controls include equivalent concentration of ceftazidime and cross-linked gelatin.

Discussion

The current study has developed glycopeptide and lipopeptide-eluting hemostatic agents that are suitable in the prevention of SSIs. This functionality is accomplished through the use of carbodiimide chemistry to create cross-linked gelatin cages that entrap antibiotics for immediate release while allowing for direct antibiotic conjugation with gelatin, providing a delayed release. These antibiotic-eluting hemostatic agents are novel in their approach to long-term release of antibiotics within surgical sites through the use of EDC for gelatin cross-linking in addition to specific conjugation of the antibiotic to the gelatin. The combination of commonly utilized surgical aids, gelatin as a hemostatic agent, and vancomycin as a highly effective antibiotic against MRSA, yields an efficient and safe method for delivering both to assist in the prevention of SSIs.

Blocking steps were removed from traditional gelatin-conjugation methods to allow for gelatin to cross-link and potentially entrap vancomycin within gelatin "cages" in addition to directly conjugating. Both type A gelatin and type B gelatin were used to produce conjugates under this method. Type A gelatin-based products were large, jelly, and sponge-like matrices that trapped large amounts of solution whereas type B gelatin-based products were smaller, more powder-like configurations that enclosed minimal amounts of solutions within them. This is likely related to a higher percentage of acidic moieties being present in gelatin type B than in gelatin type A. The alkaline processes used in type B synthesis lead to increased occurrences of glutamic and aspartic acid as opposed to the acidic processes used during type A synthesis that greatly reduces their prevalence (**Table 1**). Because powder-form hemostatic agents are stored and transported more easily, are more efficient, and are more commonly used following surgery, subsequent studies utilized type B gelatin during conjugation procedures.

Through release studies with varying concentrations of gelatin (**Figures 7 and 8**), it was seen that the use of lower concentrations of gelatin produced conjugates that released more vancomycin earlier, while the use of higher concentrations of gelatin led to conjugates that held on to the vancomycin for longer periods of time. This positive correlation between gelatin concentration and vancomycin release time is likely due to two reasons. First, increased rates of gelatin cross-linking with increasing gelatin concentration allows for vancomycin to be trapped within more complex gelatinous cages. Secondly, increased presence of gelatinous amines and carboxylic acids for vancomycin to conjugate with lead to increased rates of direct conjugation between vancomycin and gelatin. The structural integrity of released vancomycin from these products was confirmed through IR spectroscopy (**Figures 13 and 14**). This suggests that the chemical structure of released vancomycin was not significantly altered through the conjugation protocol and would likely maintain appropriate antimicrobial activities.

In order to gain more specific data on the effect of gelatin concentration on conjugate release rates, the release profile from vancomycin-gelatin conjugates with additional variations in the concentration of gelatin (**Figure 9**) was analyzed. It was observed that lower concentrations of gelatin led to higher release rates earlier in the study while higher concentrations led to higher release rates later in the study. This is consistent with previous observations and suggests that more moderate gelatin concentrations produce the most consistent rate of vancomycin release over time. Beyond release rates, it was observed that the stability, in terms of initial thickness and longevity, of the conjugates was directly related to the amount of reactant gelatin.

Although EDC-catalyzed cross-linking within gelatin has been shown to be biocompatible within biological systems without exhibiting toxicity or adverse side-effects (*79*), limited use of EDC within synthesis allows for greener and more economical synthesis and would likely expedite

FDA approval. However, lower concentrations of EDC during synthesis was expected to decrease stability and release time periods for produced conjugates. The reactant concentration of EDC, therefore, must be balanced between competing forces. As such, the effect of varied EDC concentrations (0.25x, 0.5x, 0.75x, and 1x from 50 mM standard) on release profiles from the conjugate was analyzed (**Figure 10**). It was seen that generally lower concentrations of EDC led to higher release rates earlier in the study while higher concentrations led to higher release rates later in the study. This is likely due to lower levels of EDC leading to less cross-linking of gelatin and less conjugation between gelatin and vancomycin than higher concentrations. Interestingly, at half of the original EDC concentration (25 mM), the highest release rates were seen throughout the study. This may function as a sweet-spot for elution, but repeated analysis should be performed to confirm this phenomenon.

Additionally, time-release profiles for conjugates synthesized with 20 mg/mL gelatin were determined at physiological temperature (37°C) to mimic internal use of the hemostatic agents. It was seen that release rates of the antibiotics were substantially increased over time (**Figure 11**). Higher temperatures increase the movement of molecules, leading to an increased likelihood of more antibiotic molecules breaking free of the gelatin cages and an increase in the rate of reaction for peptide bond dissociation. This would lead to more of the conjugated antibiotic being released as was observed. Additionally, the conjugates degraded more quickly at these higher temperatures as expected considering the previous discussed increased rate of reaction. Slight degradation was noted after one-week, partial degradation after two weeks, and complete degradation at the end of three weeks. Due to the likelihood of internal use of these conjugates, it was decided that reactant concentrations of gelatin should be maintained at 20 mg/mL or above to allow for extended release and appropriate hemostatic activity. The structural integrity of released antibiotics at this higher

temperature was confirmed through IR analysis suggesting that their antibacterial activity is maintained at an increased temperature (**Figure 15**).

To ensure that vancomycin released from conjugates maintained antibacterial activity, Kirby-Bauer tests were run with conjugates with 16.67 mg/mL gelatin loaded with both 2 mg/mL and 3 mg/mL gelatin against *S. Aureus* (**Figure 17**). It was seen that both samples had a zone of inhibition that extended outwards preventing bacterial growth. This confirms that the vancomycin released from the conjugates maintained its antibacterial activity. This test will be further utilized to optimize synthetic conditions. Another Kirby-Bauer test was run in triplicate for conjugates with 20 mg/mL gelatin loaded with 3 mg/mL gelatin against *S. Aureus* (**Figure 18**). It was seen that the zone of inhibition for the conjugates (22 mm average) was only slightly below that of 0.1 mg/mL pure vancomycin (25.67 mm average) which is substantially above the MIC for vancomycin (*16-17*). These tests suggest that the conjugates would have the necessary level of activity for clinical relevancy.

When considering the surface features of the hemostatic agents (**Figure 16**), the conjugates appeared to have a compact glassy layered morphology. There were slight differences between the vancomycin and daptomycin-linked hemostatic agents as the vancomycin conjugates demonstrated increased surface variations and “pitting” in comparison to the smoother morphology of the daptomycin conjugates. This is likely due to the increased availability of potential binding groups in the structure of daptomycin (**Figure 6**) as compared to vancomycin (**Figure 4**), which is likely to lead to increased rates of daptomycin-gelatin conjugation and decreased rates of gelatin-gelatin conjugation. The high levels of cross-linked fibers in the vancomycin-based conjugates are evidence of the cage-like structure of the conjugates that allow for a quicker release.

The implications of these structural differences were evident in the release profiles observed from the conjugates through HPLC (**Figure 12**). Initially, it was seen that the vancomycin-based conjugates released quickly in an exponential fashion whereas the daptomycin-based conjugates released in a slower, linear fashion. This is likely due to the presence of higher amounts of free vancomycin entrapped within the vancomycin-based conjugates as opposed to higher rates of conjugated daptomycin within daptomycin-based conjugates. This would likely occur because vancomycin (**Figure 4**) contains three less carboxylic sites for conjugation than daptomycin (**Figure 6**). In later time periods, vancomycin release slows down as daptomycin release increases. The antibiotics being released at these later time points were most likely initially conjugated to gelatin further suggesting that more daptomycin conjugated to the gelatin.

Additionally, HPLC data (**Figure 12**) indicated the presence of various species in the released samples as illustrated by a widened peak for the released antibiotics at later time points. This is likely due to released antibiotics either being conjugated to themselves or to short amino acid sequences derived from gelatin. Due to this phenomenon, some of the eluted antibiotics that are recognized in UV-Vis readings (**Figure 11**) may not be recognized in the HPLC readings leading to the observed lower concentrations within the HPLC data. UV-Vis examines the entire sample at one time in one compartment, whereas HPLC separates the sample based on size. This may cause some of the larger antibiotics conjugations to be eluted too late within the sample to be recognized as eluted antibiotics.

To ensure that the antibiotics released at later time points still maintained activity despite possible self-conjugation and gelatin presence, microdilution assays were performed at both 48 hours and 2 weeks (**Figure 19**). It was seen that both exhibited comparable and adequate activity against *S. Aureus*. Taken together with Kirby-Bauer assays, it was indicated that despite possible

variation in the structure of released antibiotics within later release samples, the released vancomycin maintains efficacy and would likely be able to be utilized within a clinical setting. Further experimentation, such as mass spectroscopy analysis, will be conducted to elucidate the structure of the various antibiotic conjugations that are released from the hemostatic agents and determine if the active site of vancomycin (**Figure 1**) is still available to inhibit bacterial cell wall formation.

While vancomycin and gelatin have been separately shown to be minimally toxic to mammalian cells (*55, 80*), the combination of the two components within the presence of EDC has not. To ensure that the presence of EDC within the conjugation procedures did not cause the produced conjugates to have toxic effects, cellular viability tests were performed (**Figure 20**). The cellular viability of all samples was comparable to untreated healthy cells illustrating that the antibiotic released from the hemostatic agent is nontoxic to mammalian cells. This is possible despite the high levels of toxicity exhibited by EDC alone, as the synthetic procedures used to generate the conjugates incorporated differences in solubility to minimize free or unreacted EDC in the final product, thereby ensuring biocompatibility of the antibiotic-eluting hemostatic agent. Additionally, studies in which gelatin was complexed with beta-TCP via various concentrations of EDC, showed that minimal concentrations of EDC (up to 10 mg/mL) were negligibly cytotoxic to cultured cells (*56*).

In comparison to vancomycin and daptomycin release (**Figure 12**), the release of the cephalosporins (**Figure 21**) seem to be much lower. Although this data is preliminary, the slower release may be due to the size of the cephalosporins relative to the glycopeptide or lipopetide antibiotics which are more than three times the size. In addition, there is the possibility that the reactivity of the cephalosporins with gelatin is significantly higher in the presence of EDC. The

structural integrity of released antibiotics is maintained according to IR spectroscopy (**Figure 22**). Cellular viability of released ceftazidime (**Figure 23**) was decreased slightly when compared to untreated healthy cells. While there is decrease, it is likely that the conjugates may still have a minimal impact on cell viability as other conjugates, pure antibiotics, and cross-linked gelatin exhibit limited influence. The recorded data may have been impacted by residual ethanol remaining in the release samples and further experimentation should be performed with more extensive drying steps.

Conclusion

Ultimately, this investigation reveals a methodology for the generation of antibiotic-eluting, gelatin-based hemostatic agents. These antibiotic-eluting hemostatic agents are novel in their approach to long-term release of antibiotics within surgical sites through the use of EDC for gelatin cross-linking in addition to specific conjugation of the antibiotic to the gelatin, yielding a continuous release of antibiotic greater than MIC concentrations but lower than toxicity levels (*16-19, 81*). Release studies show that the antibiotic is continuously released for a minimum of three weeks which exceeds the release previously shown for vancomycin from various scaffolds (*54-56*). This functionality is accomplished through the use of carbodiimide chemistry to create cross-linked gelatin cages to entrap the antibiotic for immediate release while allowing for direct antibiotic conjugation with gelatin, providing a delayed release. The combination of commonly utilized surgical aids, gelatin and vancomycin, yields an efficient and safe method for delivering both a hemostatic agent and an antibiotic to aid in the prevention of SSIs.

Beyond release, IR spectroscopy has confirmed structural integrity of released vancomycin suggesting that antimicrobial activity is maintained in these agents. The Kirby-Bauer test and microdilution assays further confirmed the activity of released antibiotics. Cell viability assays show that the conjugates have comparable biocompatibility to the released antibiotics alone. Clinical use of the antibiotic-eluting hemostatic agents developed in this study is highly accessible due to the incorporation of commonly used agents within synthesis. Gelatin is marketed as a commercially available hemostatic agent under the name of Gelfoam, while vancomycin and daptomycin are available in the solid form through many pharmaceutical companies. All together, these results suggest that this product can be incorporated into antimicrobial prophylaxis procedures to limit the occurrence of SSIs following surgery reducing the associated cost burdens

and increased mortality rates of these infections. This methodology can be further utilized to develop an assortment of products including bandages, suture replacements, and additional hemostatic agents which require a slow-release of drugs that exhibit the required functional groups.

Future Research

To craft a more complete picture of the potential of vancomycin-eluting hemostatic agents to minimize rates of infection following surgery, several more tests should be performed. Release profiles should be drawn out beyond three weeks until complete degradation of the hemostatic occurs to discover the total release of vancomycin by the hemostatic agents. Microdilution assays should then be performed at the last time point of release to ensure antibacterial activity throughout the lifetime of release. Electron microscopy should be performed on both the synthesized hemostatic agent and cross-linked gelatin for direct comparison of the two structures. Kirby-Bauer and microdilution assays should be expanded to include other bacteria such as *C. difficile* or *S. epidermidis* to allow for a broader perspective of activity. Finally, cellular viability assays should be performed with human skeletal muscle and osteogenic cells to allow for a more specific analysis of biocompatibility.

Additional tests, including those that have been performed for vancomycin, are necessary to allow for a more complete analysis of hemostatic agents synthesized with daptomycin, ceftazidime, and ceftibuten. This will allow for direct comparison on multiple levels of conjugates synthesized with antibiotics of various chemical structures to elucidate the effects of antibiotic size and existing groups on release, activity, biocompatibility, and structure of conjugates. *E. Coli* should be used in place of *S. Aureus* in Kirby Bauer and microdilution assays with the cephalosporins to demonstrate activity of conjugates against gram-positive bacteria. Following this analysis, conjugates could be synthesized with both an antibiotic targeted for gram-positive activity and an antibiotic targeted for gram-negative activity and tested on both sets of bacteria to allow for broad spectrum activity from a single conjugate.

Beyond the antibiotics currently tested, this methodology could be applied to other antibiotics containing either an amine or carboxylic moiety to allow for delayed release through both direct conjugation and gelatin cross-linking. This methodology can also be used with antibiotics that do not contain these groups but will only entrap the antibiotics through gelatin cross-linking which will likely expedite release and shorten release periods substantially. In this way, antibiotics can be optimized considering the surgical procedure, hospital environment, and the patient's background, allergies, and pre-existing conditions. Additionally, this protocol can be used to integrate chemicals with anti-inflammatory properties into gelatin-based hemostatic agents to allow for the release of these chemicals and the reduction of pain and swelling following surgical procedures.

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